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Article

Stingless bee propolis, metformin, and their combination alleviate diabetic cardiomyopathy

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Background and aim: Stingless bee propolis, a resinous compound processed by mandibular secretion of stingless bees, is used for maintenance of hygiene and stability of beehives. Research on stingless bee propolis shows therapeutic properties attributed to polyphenols exhibiting antioxidative, antihyperglycemic and antiischemic effect. However, the cardioprotective effect of stingless bee propolis on diabetic cardiomyopathy is unknown. Methods: Adult male Sprague Dawley rats were randomised to five groups: normal group, diabetic group, diabetic given metformin (DM+M), diabetic given propolis (DM+P) and diabetic given combination therapy (DM+M+P) and treated for four weeks. Body weight, fasting blood glucose, food and water intake were taken weekly. At the end of experiment, biomarkers of oxidative damage were measured in serum and heart tissue. Antioxidants in heart tissue were quantified. Part of left ventricle of heart was processed for histological staining including Haematoxylin and Eosin (H&E) stain for myocyte size and Masson's Trichrome (MT) stain for heart fibrosis and perivascular fibrosis. Results: Propolis alleviated features of diabetic cardiomyopathy such as myocyte hypertrophy, heart fibrosis and perivascular fibrosis associated with improvement in antioxidative status. Conclusion: This study reports beneficial effect of propolis and combination with metformin in alleviating histopathological feature of diabetic cardiomyopathy by modulating antioxidants, making propolis an emerging complementary therapy.

Keywords: Stingless bee. Propolis. Antioxidant. Diabetic cardiomyopathy. Endogenous secretory receptor for advanced glycation end products. Advanced Glycated End Products.

ABBREVIATION

AGE Advanced glycation end products
CAT Catalase
esRAGE Endogenous secretory receptor for advanced glycation end products
GPX-1 Glutathione peroxidase 1
MDA Malondialdehyde
SOD Superoxide dismutase

INTRODUCTION

Diabetes mellitus is the most common endocrine disorder globally and manifests as hyperglycaemia leading to metabolic abnormalities. Hyperglycaemia has direct and indirect effects on cellular metabolism and eventually generation of reactive oxygen species leading to oxidative stress (Brownlee, 2005). Oxidative stress will cause oxidative damage to cell and tissue. Diabetes mellitus is associated with cardiac changes termed diabetic cardiomyopathy, independent of hypertension

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and coronary heart disease (Miki *et al*., 2013). Diabetic cardiomyopathy entails several pathological structural changes including myocyte hypertrophy, interstitial fibrosis and perivascular fibrosis (Sharma, McNeill, Verma, 2006). The mechanism surrounding diabetic cardiomyopathy is complex, with oxidative stress and advanced glycation end products (AGE) implicated in its pathophysiology (Goldin *et al*., 2006). First line intracellular enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase 1 (GPX-1) and catalase (CAT) that offer protection against oxidative stress is affected by diabetes mellitus (Szaleczky *et al*., 1999). In addition, a protective decoy receptor named endogenous secretory receptor for advanced glycation end products (esRAGE) as the name suggests, is produced by numerous cells and serves to bind excess AGE intracellularly and extracellularly (Yonekura *et al*., 2003). Diabetes mellitus accelerates formation of AGE and reduces esRAGE through renal excretion of bound esRAGE. Therefore, pharmacological agents and natural products that restore antioxidants and esRAGE in diabetes mellitus are very much sort after.

In preclinical studies of heart, metformin can improve electrical conductivity, energy metabolism, ischemic reperfusion injury, cardiac remodeling, myocardial fibrosis and prevent cardiac hypertrophy (Mohan et al., 2018). With these cardioprotective effect of metformin, it is justified for metformin to be selected as positive control in our study. Whereas propolis is a sticky and aromatic resin formed when worker bees forage crop exudate and mixing with mandibular secretion (Jalil, Kasmuri, Hadi, 2017). This natural bee product is a commodity in ancient era due to its medicinal and therapeutic cure for multiple diseases (Kuropatnicki, Szliszka, Krol, 2013). The nature of stingless bee that does not sting and easy to manipulate by beekeeper renders meliponiculture a blooming business. Research on stingless bee propolis revealed antihyperglycaemic, antioxidative and antiischemic properties mainly attributed to polyphenols (Ahmed *et al*., 2017; Usman, Bakar, Mohamed, 2016). Among the stingless bee species notable in Malaysian meliponiculture, *Heterotrigona Itama* propolis showed better antioxidative activity (Ibrahim *et al*., 2016). However, the effect of *Heterotrigona Itama* propolis in diabetic cardiomyopathy was yet to be investigated. The antioxidative and antihyperglycaemic effect of Itama propolis is postulated to prevent diabetic cardiomyopathy. Therefore, this study is set to determine the histopathology of heart, its associated biochemical changes such as oxidative damage marker (MDA), antioxidants (SOD, GPX-1, CAT) and the interaction of AGE and esRAGE in serum and heart after propolis or metformin supplementation or combination of both in diabetic rats.

MATERIAL AND METHODS

Ethanolic extraction of propolis

Raw propolis (*Heterotrigona Itama*) was obtained from Min House Camp (6.090214, 102.291119) Kota Bharu, Kelantan, Malaysia. Extraction of propolis by ethanol is in accordance with a published protocol gives higher antioxidative activity and extraction yield (Usman, Bakar, Mohamed, 2016). The propolis was cleaned, grounded and macerated in 70% ethanol before being filtered. The filtrate was concentrated, freeze dried and stored at -20 ^{o}C for further study.

Experimental animals

Forty adult male Sprague-Dawley rats aged 10-12 weeks weighing 190-220g were used in this study. The animals were acclimatised in an animal room with temperature of 25± 2 $^{\circ}$C and 12 hours light-dark cycle for a week. The rats have access to drinking water and standard pellet *ad libitum*. The study was approved by Universiti Sains Malaysia Institutional Animal Care and Use Committee [USM/IACUC/2018/(112)] and was conducted with strict adherence to animal ethics guidelines after approval. Upon receipt, rats were acclimatised for a week with free access of water and normal pellet diet. After acclimatisation, the animals were divided into five groups (n=8/group) randomly as follows:

Group 1: normal healthy group (NG).
Group 2: diabetes group (DM).

Group 3: diabetic given metformin (DM+M) treated with 300mg/kg metformin per day. Group 4: diabetic given propolis (DM+P) treated with 300mg/kg propolis extract per day. Group 5: diabetic given combination of metformin and propolis (DM+M+P) treated with 300mg/kg metformin and 300mg/kg propolis extract per day based on previous study (Usman *et al*., 2017).

Single dose of streptozotocin (60 mg/kg) was given intraperitoneally to induce diabetes (Gajdosik *et al*., 1999) in group 2-5. Whereas rats in normal group (NG) were injected one millilitre of normal saline intraperitoneally as vehicle. The attainment of diabetes mellitus was confirmed by fasting blood glucose of tail vein blood more than 200 mg/dl on the third day after induction of diabetes (Qinna and Badwan, 2015). Treatment was administered for four weeks using oral gavage after successful induction of diabetes. Throughout the four weeks experimental period, the rats' body weight, water intake, food intake and fasting blood glucose were recorded on weekly basis. Propolis and metformin dosage administered was based on previous study (Nna *et al*., 2018). After four weeks of treatment, the animals were anesthetised with 200 mg/kg sodium pentobarbital for sacrifice before dissection (Close *et al*., 1997). 5 millilitre of blood sample was aspirated from inferior vena cava of each rat for measurement of fasting blood glucose. Blood samples were allowed to coagulate at room temperature before being centrifuged for 10 minutes to yield serum. Immediately, heart tissue was excised, washed three times with ice cold phosphate buffer saline and weighted. Left ventricle of heart tissue samples were stored in 10% formalin as fixative solution for histopathological examination. The remaining heart tissue was processed into 10% tissue homogenate.

QUANTIFICATION OF HOMOGENATE AND SERUM BIOCHEMICAL MARKERS

Heart tissue homogenate were centrifuged and the resulting supernatant was fed into assays. Commercially available kits were used to measure levels of protein concentration (BCA Protein Assay Kit, AR0146, Boster Biological Technology, USA), malondialdehyde (MDA) (E-EL-0060, Elabscience®, USA), superoxide dismutase (SOD) (E-BC-K020, Elabscience®, USA), glutathione peroxidase 1 (GPX-1) (E-EL-R2491, Elabscience®, USA), catalase (CAT) (E-BC-K031, Elabscience®, USA), Rat Endogenous Secretory Advanced Glycosylation End Product Specific Receptor (esRAGE) (E-EL-R2497, Elabscience®, USA) and Advanced Glycation End Product (AGE) (STA-817, OxiSelect™, Cell Biolabs, USA), in rats' heart tissues. Serum was used to quantify esRAGE and AGE with the kit mentioned previously. The experimental procedures were strictly adhered to the protocol given by manufacturer. The derived ratio (SOD/CAT+GPX-1), heart/serum esRAGE, heart/serum AGE, heart AGE/heart esRAGE and serum AGE/heart esRAGE were also calculated.

Histopathological examination

The formalin-fixed heart tissue was processed in tissue processor overnight and embedded in paraffin. The paraffin-embedded tissues were cooled and sectioned into 3 μm slices using rotatory microtome. The sectioned tissues were fixed on glass slides and stained with Hematoxylin and Eosin (H&E) and Masson's Trichrome (MT) dye. Tissues stained with H&E were viewed under image microscope (Olympus BX41, Japan) at 400x magnification for qualitative analysis and quantitative measurement of myocyte size. The criteria for myocyte measurement was regular circular to oval shape and presence of nucleus. Theoretically, the cut section of cells is at transverse section with minimal angulation if the cell is circle to oval shaped and section at level of nucleus indicate central location of a cell. Myocytes that fulfill the inclusion criteria will be analysed manually using ImageJ software. In addition, MT stained tissues were visualised at 200x and 400x magnification for quantitative measurement of heart fibrosis and perivascular fibrosis respectively. All quantitative measurements were measured using ImageJ software (ImageJ, NIH-Bethesda, MD, USA). Photomicrographs were taken per biological replicate (n=8/group). Photomicrographs were taken from 5 different fields for each replicate and used for the analysis.

Statistical analysis

Oxidative stress, antioxidative and quantitative histological data were presented as mean ± standard deviation (SD). Data analysis was done using one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. Body weight, fasting blood sugar, food intake and water intake was analysed using repeated measure ANOVA followed by one-way ANOVA to identify difference between groups if statistically significant. GraphPad prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA) was used for the analyses and $p < 0.05$ was considered statistically significant.

RESULTS

Effect of propolis, metformin and combination therapy on fasting blood glucose, body weight, food intake and water intake

Over four weeks of diabetes, classical symptoms such as weight loss, hyperglycaemia, polyphagia and polydipsia were evident. Diabetic group observed significant hyperglycaemia ($p < 0.0001$) compared to normal group, propolis group, metformin group and combination group. Propolis, metformin or both significantly reduced ($p < 0.0001$) hyperglycaemia compared to diabetic group from end of week 1 onwards, signifying its sustainable antihyperglycaemic property. Interestingly, the most effective antihyperglycaemic treatment is the combination group (DM+M+P) (Figure 1). Signs of diabetes mellitus such as muscle wasting, polyphagia and polydipsia were observed in diabetic groups evidenced by weight loss (Figure 2) significantly higher food and water intake (Figure 3), and these signs were improved in treatment groups particularly in groups receiving combination therapy.

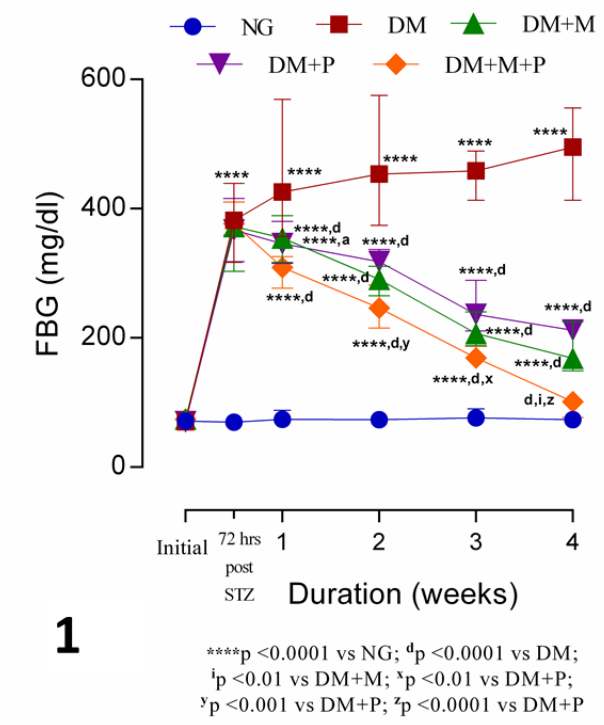

FIGURE 1 - Effect of propolis, metformin and combination on fasting blood glucose. Values are expressed as mean ± SD, n = 8. ****p<0.0001 vs NG; [d]p<0.0001 vs DM; [i]p<0.01 vs DM+M; [x]p<0.01 vs DM+P; [y]p<0.001 vs DM+P; [z]p <0.0001 vs DM+P.

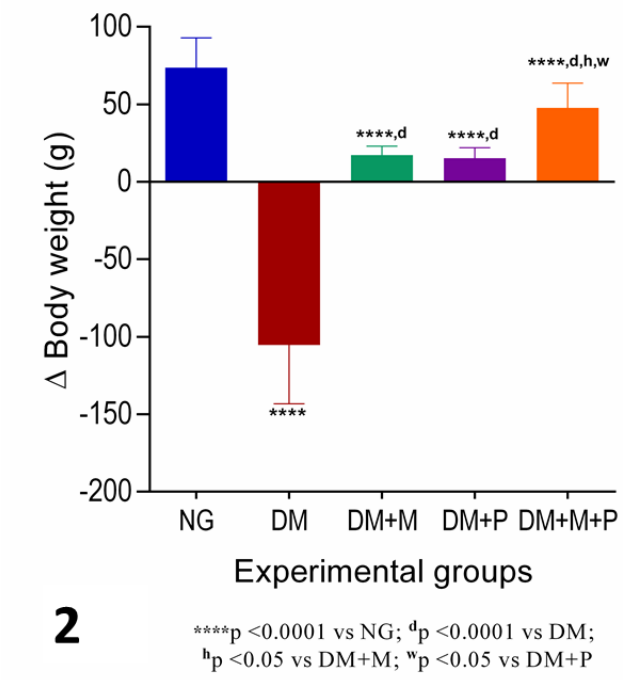

FIGURE 2 - Effect of propolis, metformin and combination on change in body weight. Values are expressed as mean ± SD, n = 8. ****p<0.0001 vs NG; [d]p<0.0001 vs DM; [h]p<0.05 vs DM+M; [w]p<0.05 vs DM+P.

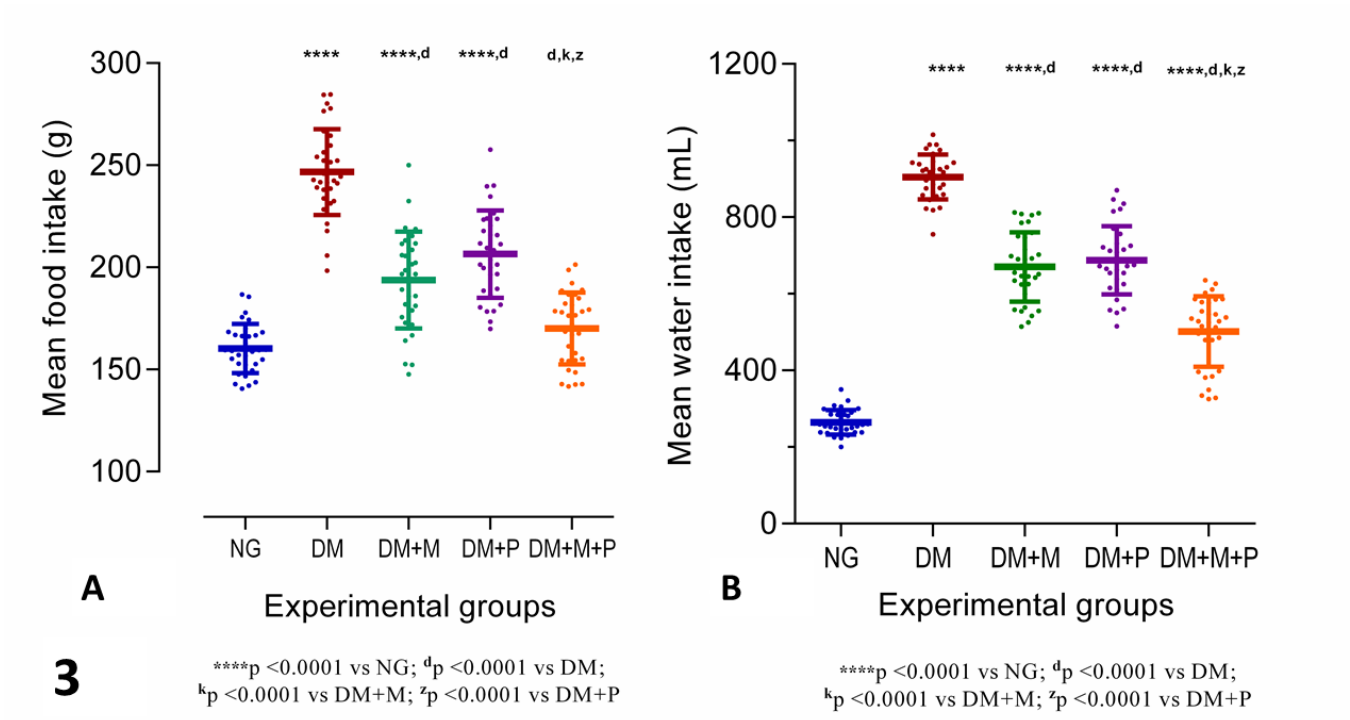

FIGURE 3 - Effect of propolis, metformin and combination on food intake and water intake. Values are expressed as mean ± SD, n = 8. ****p<0.0001 vs NG; [d]p<0.0001 vs DM; [k]p<0.0001 vs DM+M; [z]p<0.0001 vs DM+P.

Effect of propolis, metformin and combination therapy on cardiac antioxidant enzymes activities and MDA

Hyperglycaemia causes modulation in antioxidative activities and oxidative stress in heart tissue. Regarding antioxidative defense enzymatic activity, there were significant differences in diabetic group SOD ($p < 0.05$), GPX-1 ($p < 0.001$) and CAT ($p < 0.05$) compared to normal group (Table I). Metformin and propolis monotherapy significantly raised ($p < 0.05$) SOD activity in diabetic rats. However, combination group observed significant antagonism ($p < 0.05$) in SOD activity compared to metformin treated group. All treatment groups had higher GPX-1 concentration relative to DM, and best seen in propolis treated group ($p < 0.01$). All treatment group had lower CAT activity relative to DM and best seen in DM+M+P combination group ($p < 0.01$). The malondialdehyde (MDA) level in DM group was higher than NG but not reaching statistical significance, suggesting lipid peroxidation from oxidative stress. Propolis and combinatin therapy had lower MDA level compared to DM.

Effect of propolis, metformin and combination therapy on composite oxidative stress ratio in heart SOD/GPX-1, SOD/CAT, SOD/(GPX-1+CAT)

The SOD/GPX-1 ratio in DM was raised relative to NG, among the treatment groups, DM+M had higher SOD/GPX-1 ratio than DM, DM+P and DM+M+P had lower SOD/GPX-1 than DM (Table I). It is worth noting that metformin monotherapy caused more oxidative stress as shown in higher SOD/GPX-1 ratio relative to DM which is significantly reduced ($p < 0.01$) when given together with propolis, thereby protecting heart from hydrogen peroxide causing oxidative stress. In contrast, DM had lower SOD/CAT ratio than NG, most likely compensatory changes to remove excess hydrogen peroxide. SOD/(GPX-1+CAT) ratio was used to reflect oxidative stress from hydrogen peroxide accumulation in heart. DM had higher SOD/(GPX-1+CAT) ratio compared to NG, only combination of metformin and propolis therapy (DM+M+P) showed significantly lower ($p < 0.05$) SOD/(GPX-1+CAT) ratio compared to DM, suggesting its ability to reduce oxidative stress from accumulation of hydrogen peroxide. Metformin monotherapy had significantly higher SOD/(GPX-1+CAT) ratio relative to propolis monotherapy ($p < 0.01$) or combination of both ($p < 0.001$).

Effect of propolis, metformin and combination therapy on heart AGE, serum AGE, heart esRAGE, serum esRAGE concentration and their composite ratio

Advanced glycation end products (AGE) is present in heart and serum, when present in high concentration will lead to deleterious effects to heart. No significant changes in the serum or cardiac AGE was observed, but heart/serum AGE ratio was higher in diabetic group, suggesting higher accumulation of AGE in heart than in the serum. As for esRAGE, significant changes ($p < 0.05$) were seen in propolis treated group (Table I), suggesting a novel cardioprotective property in diabetic rats. All treatment groups demonstrated increase in heart/serum esRAGE compared to DM, suggesting increased concentration of protective decoy receptor esRAGE in heart relative to serum. esRAGE binds to excess AGE and removes AGE, thus the ratio of AGE/esRAGE may serve as a biomarker in diabetic cardiomyopathy. Combination of propolis and metformin observed significant changes ($p < 0.05$) in heart AGE/esRAGE ratio, suggesting synergistic action in preventing diabetic cardiomyopathy.

TABLE I - Effect of propolis, metformin and combination therapy on cardiac and serum antioxidants and oxidative stress

Parameters	NG	DM	DM+M	DM+P	DM+M+P
SOD (U/mg)	2.38 ± 0.38	1.37 ± 0.58 *	2.41 ± 0.69 a	2.20 ± 0.24 a	1.56 ± 0.60 *h
GPx-1 (µg/mg)	1181 ± 255	686 ± 246 **	969 ± 193	1123 ± 212 b	1068 ± 199 a
CAT (U/mg)	0.43 ± 0.02	0.58 ± 0.09 *	0.52 ± 0.09	0.51 ± 0.12	0.39 ± 0.10 b
MDA (ng/mg)	0.55 ± 0.06	0.87 ± 0.28	0.94 ± 0.21	0.75 ± 0.19	0.64 ± 0.31
SOD/GPX-1	1.98 ± 0.46	2.70 ± 1.16	2.99 ± 0.97	2.02 ± 0.47	1.55 ± 0.77 h
SOD/CAT	5.51 ± 0.91	3.13 ± 1.33 *	5.51 ± 2.41 a	4.49 ± 1.02	3.91 ± 0.98
SOD/(GPX-1+CAT)	1.45 ± 0.28	1.60 ± 0.38	2.07 ± 0.42 a	1.36 ± 0.22[i]	0.96 ± 0.34 a,j
Heart AGE (µg/mg)	1.30 ± 0.10	1.49 ± 0.24	1.57 ± 0.20	1.43 ± 0.12	1.37 ± 0.22
Heart esRAGE (ng/mg)	0.74 ± 0.32	0.20 ± 0.18 *	0.53 ± 0.26	0.66 ± 0.33 a	0.58 ± 0.19
Serum AGE (µg/mL)	321 ± 24	296 ± 43	308 ± 39	304 ± 25	298 ± 23
Serum esRAGE (ng/mL)	0.44 ± 0.05	0.55 ± 0.07	0.39 ± 0.06	0.62 ± 0.13 h	0.52 ± 0.24
Heart/Serum AGE	4.09 ± 0.49	5.16 ± 1.07	5.15 ± 0.82	4.74 ± 0.52	4.43 ± 1.02
Heart/Serum esRAGE	1.78 ± 1.02	0.39 ± 0.42**	1.03 ± 0.41	0.88 ± 0.30	1.16 ± 0.65
Heart AGE/esRAGE	4.77 ± 2.47	1.74 ± 0.50*	3.14 ± 1.46	2.50 ± 0.91	2.09 ± 0.52 a
Serum AGE/esRAGE	0.72 ± 0.16	0.55 ± 0.14	0.74 ± 0.16	0.55 ± 0.07	0.60 ± 0.24

Values are mean ± SD, n = 8. *p<0.05, **p<0.001 vs NG; ap<0.05, bp<0.01 vs DM; hp<0.05, ip<0.01, jp<0.001 vs DM+M (One-way ANOVA, followed by Tukey post-hoc test).

Qualitative changes in myocardium

Histology of the heart at cross section of myocyte on 400x magnification revealed non-specific changes such as widespread cellular disarray, pyknotic nuclei, cytoplasmic vacuolation, necrotic myocyte and infiltration of inflammatory cells between adjacent myocytes in DM compared to NG (Figure 4B, 5B). Administration of metformin was associated with slight improvement in the histological changes while administration of propolis and combination of treatment lead to significant improvement in the diabetic-induced pathological changes in myocardium (Figure 4). Longitudinal orientation of myocytes in DM showed wavy arrangement with disorganised branching and hypertrophied striated muscle compared to NG. DM+M, DM+P and DM+M+P improved the pathological changes in diabetic myocardium (Figure 5).

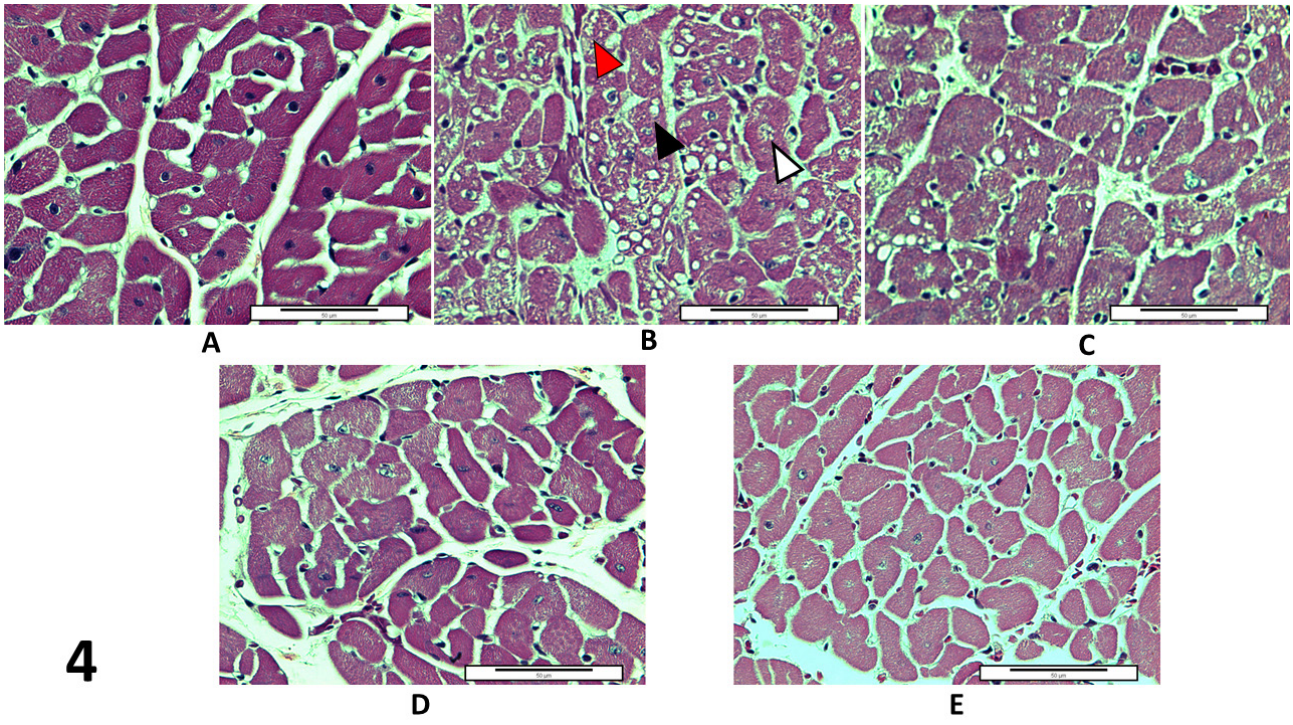

FIGURE 4 - Representative photomicrograph of transverse section of rat myocardium in left ventricle viewed under 400x magnification with Haematoxylin and eosin stain of NG (A), DM (B), DM+M (C), DM+P (D) and DM+M+P (E). The pathological changes in DM such as pyknotic nuclei (empty arrow), cytoplasmic vacuolation (solid black arrow) and necrotic myocyte (solid red arrow) were shown.

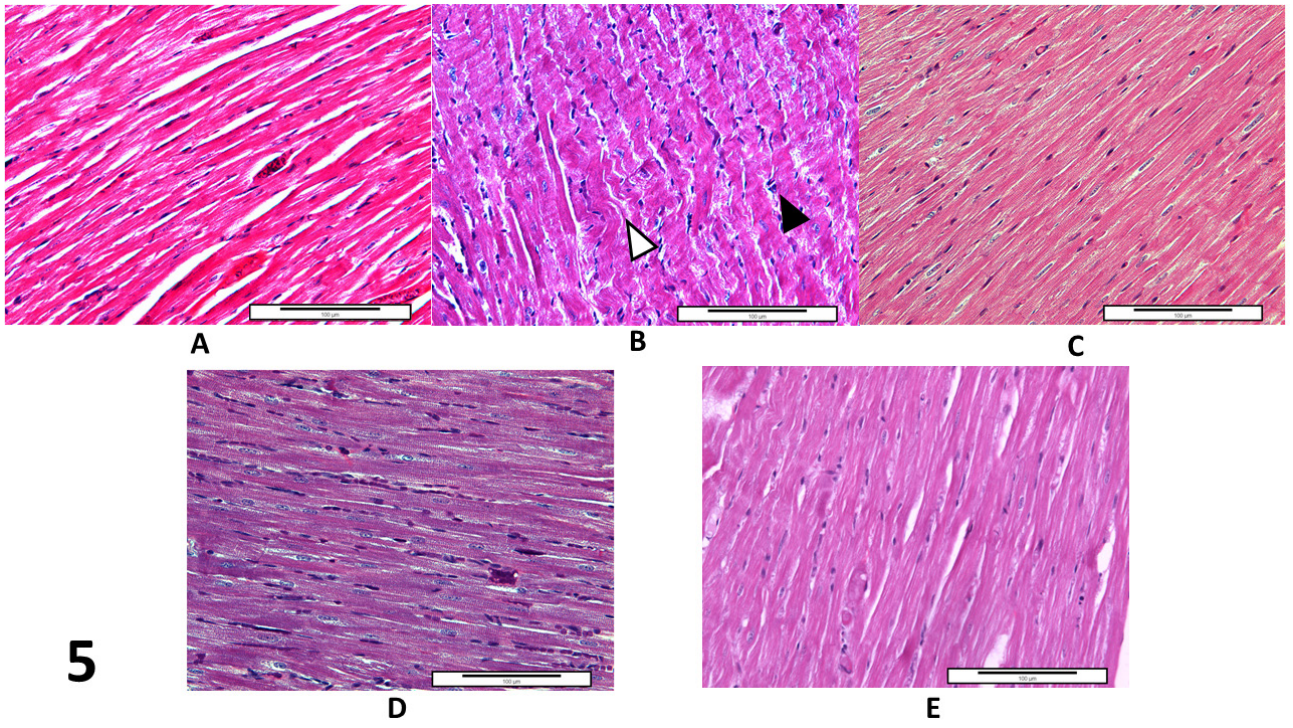

FIGURE 5 - Representative photomicrograph of longitudinal orientation of rat myocardium in left ventricle viewed under 200x magnification with Haematoxylin and eosin stain of NG (A), DM (B), DM+M (C), DM+P (D) and DM+M+P (E). The pathological changes in DM such as widespread cellular disarray (empty arrow) and infiltration of inflammatory cells (solid arrow).

Quantitative changes in heart histopathology

Cardiac hypertrophy

DM and DM+M had significantly lower heart weight compared to NG, DM+P and DM+M+P had significantly higher heart weight compared to DM (Figure 6A). When corrected for body weight (HW/BWx1000), DM had significantly higher HW/BWx1000 relative to NG, implying cardiac hypertrophy (Figure 6C). All treatment groups significantly reduced ($p < 0.01$) cardiac hypertrophy versus DM. There was no significant difference in myocyte size between groups (Figure 6D). When myocyte size was corrected for heart weight (myocyte size/heart weight) and body weight (myocyte size/body weight), DM demonstrated significant myocyte hypertrophy compared to NG (Figure 6E, 6F). Administration of metformin, propolis or combination of both significantly reduced ($p < 0.01$) myocyte hypertrophy.

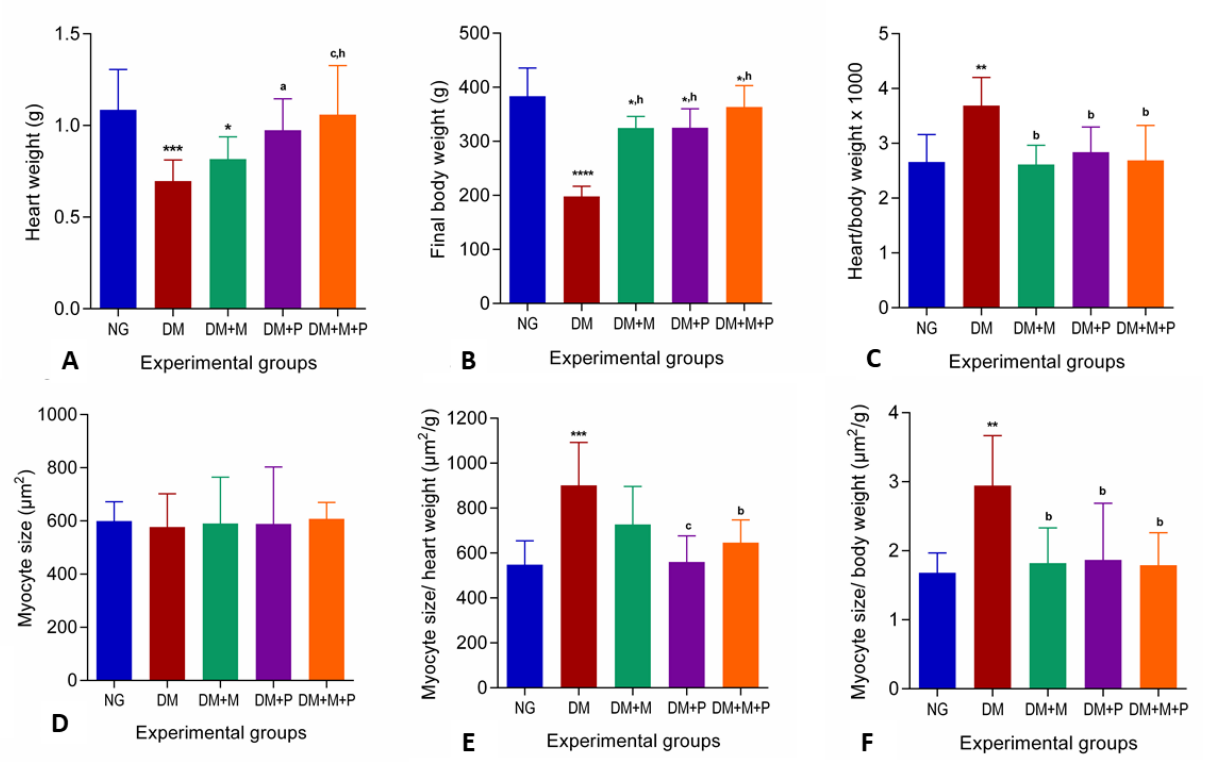

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FIGURE 6 - Effect of propolis, metformin and combination therapy on heart weight (A), final body weight (B), heart/body weight x100 (C), myocyte size (D), myocyte size/heart weight (E) and myocyte size/body weight (F). Values are expressed as mean ± SD, n = 8. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs NG; [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ vs DM; [h]$p<0.05$ vs DM+M.

Interstitial fibrosis

Interstitial fibrosis is represented by green colour highlighted for collagen deposition as main component of fibrosis using Masson's Trichrome stain (Figure 7A-E). DM showed a significant increase ($p < 0.0001$) in interstitial fibrosis compared to NG. All treatment groups DM+M, DM+P and DM+M+P significantly alleviated interstitial fibrosis compared to DM (Figure 7F). Among the treatment groups, DM+P observed the most significant reduction ($p < 0.001$) in interstitial fibrosis.

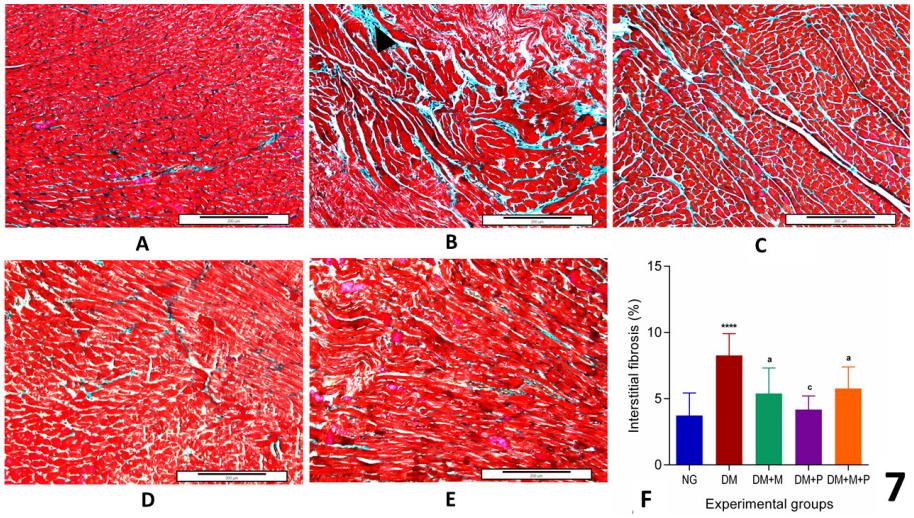

FIGURE 7 - Representative photomicrograph of rat myocardium in left ventricle viewed under 100x magnification with Masson's Trichrome stain of NG (A), DM (B), DM+M (C), DM+P (D) and DM+M+P (E). Interstitial fibrosis is represented by green colour highlighted for collagen deposition as main component of fibrosis using Masson's Trichrome stain (solid black arrow). Effect of propolis, metformin and combination therapy on interstitial fibrosis (F). Values are expressed as mean ± SD, n = 8. ****p < 0.0001 vs NG; [a]p<0.05, [c]p<0.001 vs DM.

Perivascular fibrosis

Perivascular fibrosis is obtained from the ratio of perivascular collagen area over luminal area highlighted in green as shown by solid arrow in Figure 8B. DM showed significantly higher ($p < 0.0001$) perivascular fibrosis relative to NG. Metformin (DM+M) was not significant compared to DM (Figure 8F). However, propolis (DM+P) and combination group (DM+M+P) achieved significant reduction in perivascular fibrosis ($p < 0.05$ and $p < 0.001$ respectively) compared to DM.

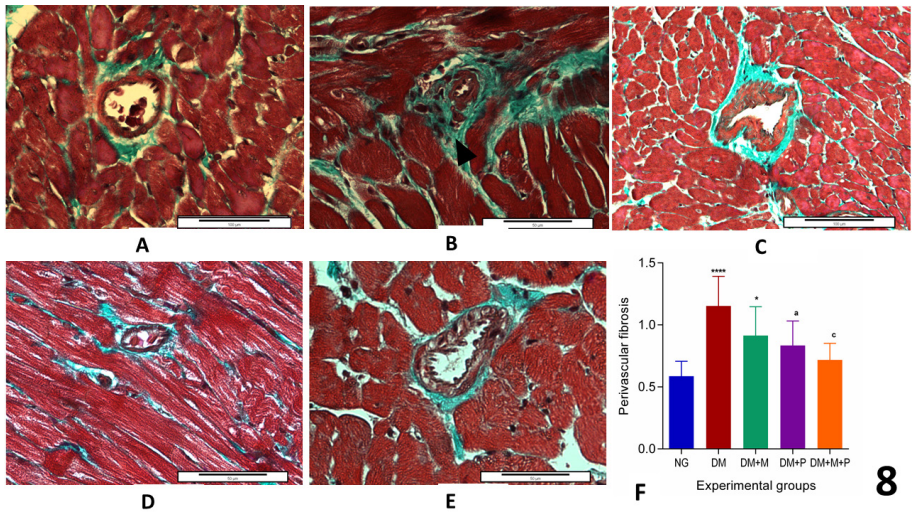

FIGURE 8 - Representative photomicrograph of a microvessel in rat myocardium viewed under 400x magnification with Masson's Trichrome stain of NG (A), DM (B), DM+M (C), DM+P (D) and DM+M+P (E). Perivascular fibrosis is represented by green colour highlighted for collagen deposition as main component of fibrosis using Masson's Trichrome stain (solid black arrow). Effect of propolis, metformin and combination therapy on perivascular fibrosis (F). Values are expressed as mean ± SD, n = 8. *p < 0.05, ****p < 0.0001 vs NG; [a]p<0.05, [c]p<0.001 vs DM.

DISCUSSION

Previous studies pertaining to stingless bee propolis demonstrated glucose lowering effect after supplementation (Nna *et al*., 2018; Usman *et al*., 2017). Interestingly, the glucose lowering effect of stingless bee propolis does not extrapolate to rats with normal glucose, so the term anti-hyperglycaemic agent was more suitably applied than hypoglycaemic agent (Nna *et al*., 2018). In our current study, propolis exhibited cardioprotective effect on diabetic cardiomyopathy. The fact that propolis can be comparable to metformin in improving almost all parameters of symptomatic hyperglycaemia indicates the potential of propolis as an antidiabetic functional food. This is especially true when numerous countries examining propolis has concluded that propolis is an effective anti-hyperglycaemic agent (El-Sadany, 2016). Several studies also support the combined use of metformin and propolis in diabetic patients, evidenced by synergistic beneficial effect on pancreas and liver (Nna *et al*., 2018; Usman *et al*., 2017). Metformin can inhibit hunger hormone ghrelin and hunger neuron NPY besides increasing sensitivity to satiety hormone leptin in hypothalamus (Gagnon, Sheppard, Anini, 2013). On the other hand, propolis can restore satiety hormone leptin and insulin (Usman *et al*., 2017). Combination of metformin and propolis may act synergistically in prevention of polyphagia due to different site or mechanism of action.

Diabetes mellitus leads to progressive increase in fasting blood glucose seemingly out of control due to glucotoxicity, beta cell exhaustion and desensitisation (Cernea, Dobreanu, 2013). Metformin, propolis or combination of them improved fasting blood glucose in diabetes mellitus. Metformin inhibits hepatic gluconeogenesis, prevented reabsorption of glucose of the gut and improve insulin sensitivity (Bjornstad *et al*., 2018). Propolis from China, Brazil (El-Sayed *et al*., 2009), Egypt (El-Sadany, 2016) and Malaysia (Usman *et al*., 2017) possess glucose lowering effect. Malaysian propolis restored pancreatic islet function, insulin secretion, insulin sensitivity, reduced glucagon and prevented α-glucosidase activity, all of which can reduce fasting blood glucose (Usman *et al*., 2017; Nna *et al*., 2018). The bioactive component of propolis possessing anti-hyperglycaemic potential is still not identified. Loss of body weight in diabetes mellitus is replicated in previous study (Pournaghi *et al*., 2012). The discrepancy of food intake and body weight in diabetes mellitus represents negative energy balance. Metformin reverses negative energy balance by increasing glucose uptake in tissues (Beysel *et al*., 2018). Modulation of insulin by propolis accounts for the increase in body weight in diabetes mellitus (Usman *et al*., 2017; Nna *et al*., 2018). Combination of metformin and propolis once again observed synergistic effect in diabetic mellitus by restoring body weight almost similar to normal healthy group.

Diabetes mellitus affects cellular metabolism leading to oxidative stress from generation of reactive oxygen species. Antioxidants removes excess reactive oxygen species and reduces oxidative stress. High oxidative stress can have deleterious effect in cells. First line enzymatic intracellular antioxidant defense mechanism is by the collective action of superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT). Reactive oxygen species such as superoxide anion is converted to hydrogen peroxide by SOD and then to stable compound water and oxygen by GPX and CAT (Ighodaro and Akinloye, 2018). Composite oxidative stress ratio reflected the accumulation of radical compound hydrogen peroxide. Glutathione peroxidase 1 is the most common isoform of GPX in cells. Diabetes mellitus causes perturbation in intracellular antioxidants (Szaleczky *et al*., 1999). In our study on heart, DM group has lower SOD and GPX but not CAT relative to normal control group. Catalase in heart may be overexpressed and overactivated as a compensatory response towards oxidative stress (Cong *et al*., 2015). Malondialdehyde (MDA) is a biomarker of lipid peroxidation due to oxidative stress. The lack of difference between groups may due to increased catalase activity to compensate oxidative stress in diabetic group. Metformin exerts antioxidative activity mainly through SOD, propolis acts through SOD and GPX, whereas combination of metformin and propolis acts on GPX and CAT. The fact that metformin increases MDA may suggest activation of SOD may lead to accumulation of hydrogen peroxide in heart.

Composite oxidative stress as in SOD/GPX-1, SOD/CAT and SOD/(GPX-1+CAT) indicates oxidative stress from accumulation of hydrogen peroxide in heart tissue (Mladenov *et al*., 2015). DM shows lower SOD and GPx (increased SOD/GPX-1 & SOD/GPX+CAT ratio), associated with higher MDA level (although statistically not significant), probably due to relative short duration of disease state or compensatory effect from CAT (evidenced with higher CAT or SOD/CAT ratio). Metformin causes more oxidative stress reflected by increased MDA, SOD/GPX-1 and SOD/(GPX-1+CAT) compared to diabetic group. This is in contrast with other studies, probably due to early effects of metformin on heart. However, both propolis and combination of metformin and propolis observed reduction in oxidative stress such as MDA, SOD/GPX-1 and SOD/(GPX-1+CAT) and is best seen with combination of metformin and propolis. Previous study on *Itama* propolis noted in vitro antioxidative activity due to high phenolic compounds and flavonoids (Nna *et al*., 2018). Propolis may have directly scavenged the reactive oxygen species in heart leading to replenished stores of antioxidants or stimulated the increase of antioxidant activity or indirectly increased antioxidants by improving hyperglycaemia, leading to less reactive oxygen species to be scavenged by antioxidants.

Hyperglycaemia non-enzymatically glycates amino acid, lipid and nuclei acids to yield advanced glycation end products (AGE) via the Maillard reaction. The interaction of AGE with AGE receptor (RAGE) will lead to production of reactive oxygen species and oxidative stress, causing cardiomyopathy (Jia, Hill, Sowers, 2018). DM has higher AGE but our study shows that metformin increases AGE in heart, corresponding to MDA, oxidative stress SOD/GPX-1 and SOD/(GPX-1+CAT). However, serum AGE does not reach statistical significance. The rapid turnover rate of serum AGE is the most plausible reason (Gugliucci, Menini, 2014). Protective scavenging receptor, endogenous secretory receptor for AGE (esRAGE) is produced by numerous cells that binds to excess AGE and remove them (Heier *et al*., 2015). Heart in diabetic rat contains lower concentration of esRAGE and this can either be attributed to reduced production of esRAGE in heart or increased excretion of bound esRAGE-AGE molecule. All treatment groups restored esRAGE in heart with propolis treatment group achieved the best result. This cardioprotective property of propolis warrants further research on whether propolis directly stimulates production of esRAGE or indirectly increase esRAGE by reducing AGE as in improvement in hyperglycaemia. In addition, combination of propolis and metformin possess synergistic cardioprotective action in heart evidenced by reduced heart AGE/esRAGE ratio. However, serum esRAGE has to be interpreted with caution due to its dependence of renal excretion and production by many other cells such as vascular endothelial cell, monocyte, macrophage and pancreas (Heier *et al*., 2015).

In diabetes mellitus, heart undergoes non-specific pathological changes when viewed under H&E stain including cytoplasmic vacuolation, cellular disarray, degeneration, necrosis and apoptosis (Zhang, Wei, 2013). Treatment of propolis or combined propolis and metformin ameliorated the changes. The pathological hallmarks of diabetic cardiomyopathy are cardiac hypertrophy, interstitial fibrosis and perivascular fibrosis (Sharma, McNeill, Verma, 2006). Cardiac hypertrophy is due to myocyte hypertrophy or an increase in extracellular matrix (Shimizu, Minamino, 2016). The heart weight in diabetes mellitus is reduced corresponding to body weight. When heart weight is corrected for body weight (heart/body weight), the diabetic-induced cardiac hypertrophy is evident. Similar observation was seen in myocyte size. When myocyte size was corrected for heart weight, the diabetic-induced myocyte hypertrophy becomes obvious. When evaluating cardiac hypertrophy due to extracellular matrix deposition, diabetes mellitus causes significant deposition of collagen which is the main component of extracellular matrix homogenously in heart (interstitial fibrosis) and around the microvascular structure (perivascular fibrosis). Among the mechanisms implicated in pathological changes of diabetic cardiomyopathy, AGE and oxidative stress is the culprit (Jia, Hill Sowers, 2018).

Myocyte hypertrophy occurs as an attempt to increase performance in stressed condition that involves activation of fetal cardiac genes. Propolis or combination of metformin and propolis improved myocyte hypertrophy. Propolis administration can reduce

switching of fetal cardiac ANF and β-MHC genes in myocardial infarcted heart (Samak *et al*., 2016). Cardiac interstitial fibrosis and perivascular fibrosis in diabetes mellitus are due to recruitment of collagen secreting myofibroblast through activation of transforming growth factor beta (TGF-β) (Lijnen, Petrov, 2000). Metformin significantly improves interstitial fibrosis but not perivascular fibrosis. Metformin can directly inhibit collagen synthesis from myofibroblasts by inactivation of TGF-β (Burlá *et al*., 2013). Whereas, propolis or combination of propolis and metformin significantly reduce interstitial and perivascular fibrosis.

CONCLUSION

Our study reports cardioprotective effects of propolis in diabetic rats by alleviating histopathological feature of diabetic cardiomyopathy through modulation of antioxidants, making propolis an emerging complementary therapy. Combined therapy with metformin showed beneficial synergistic effects, suggesting stingless bee propolis may be a potential candidate as adjuvant therapy in diabetic patients.

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